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A Simple Procedure for Sulfation and ^{35}S Radiolabelling of Paralytic Shellfish Poisoning (PSP) Gonyautoxins

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ABSTRACT A method is described to sulfate PSP toxins at various positions in the molecule and to prepare ^{35}S labelled compounds using $H_2{}^{35}SO_4$ in the presence of dicyclohexylcarbodiimide (DCC). The 11-sulfates of saxitoxin and neosaxitoxin, known as gonyautoxins, are often the most abundant of the PSP toxins in algae and contaminated shellfish. Receptor site binding and antibody assays based on these analogues should, therefore, better reflect toxicity than those in which saxitoxin is used. Although the specific activity of ^{35}S-gonyautoxins is lower than that of commercially available ^{3}H-saxitoxin, the label is strongly bound and is not lost through proton exchange with water as occurs with tritiated saxitoxin. The labelling procedure is rapid, inexpensive and can be done on a small scale. Sulfate can be removed from the 11-position of GTX's in methanolic-HCl and from the 21-position by mild acid hydrolysis and $H_2{}^{35}SO_4$ added in 5–10-fold excess. Addition or exchange occurs rapidly on mixing DCC in dimethylformamide with dry toxin and sulfate. Reaction conditions were optimized and reaction products identified by capillary electrophoresis, autoradiography and ionspray mass spectrometry. Together with methods for selective removal of sulfate, the sulfation reaction provides an additional way to prepare some of the naturally occurring derivatives of saxitoxin, many of which are sulfates. *Nat. Toxins 5:36–42, 1997.*

Key Words: shellfish; marine toxin; saxitoxin; sulfate; radioisotope; capillary electrophoresis

INTRODUCTION

Saxitoxin and its analogues are among the most toxic low molecular weight compounds known. They are produced by some dinoflagellates and cyanobacteria and can accumulate to high concentrations in organisms that feed on them. These tetrahydropurines block sodium channels of the central nervous system causing paralytic shellfish poisoning (PSP). Because PSP is not entirely predictable, regular monitoring is necessary to protect consumers and the shellfish industry. The mouse bioassay has been used in research and fisheries protection laboratories for more than fifty years but it requires live animals and sensitivity is close to the regulatory limit of 80 μg/100 g tissue. In addition to high sensitivity, new assay methods for PSP toxins must be sufficiently selective to avoid interferences from components in the shellfish homogenate, yet encompass the range of properties of the more than 20 natural saxitoxin analogues (Fig. 1). HPLC methods are currently the most widely used with either postcolumn [Sullivan et al., 1988; Oshima et al., 1989] or precolumn oxidation to fluorescent derivatives [Lawrence and Ménard, 1991]. Alternatively, antibody [Usleber et al., 1995] and receptor site assays using neuroblastoma cells [Jellett et al., 1995; Manger et al., 1995] or isolated sodium channels [Vieytes et al.,1993] both show promise and do not require expensive equipment.

Fluorescent oxidation products are usually used to detect PSP toxins after chromatography. However, alternative detection methods are necessary with antibody and receptor site methods. These are often competition assays in which a known amount of a labelled toxin is used. Fluorescent or radiolabelled toxin is then displaced from the antibody or receptor by toxins in the extract. Antibody methods can incorporate an enzyme to produce a measurable color. These enzyme linked immunosorbent assay (ELISA) methods avoid the use of radioactive materials but radiolabelled antigens are useful in their development. Tritiated saxitoxin has been the only radiolabelled PSP toxin available to date [Ritchie et al., 1976]. A major disadvantage of this material, however, is the rapid exchange of tritium for hydrogen in aqueous solutions, which limits it usefulness in assays.

The 11-sulfates (gonyautoxins, or GTXs) are probably the most common of all of the PSP toxins. GTX2 and GTX3 are α and β epimers, respectively, of saxitoxin 11-sulfate; and GTX1 and GTX4 are the α and β epimers, respectively, of neosaxitoxin 11-sulfate. Toxicities of the GTX's are similar

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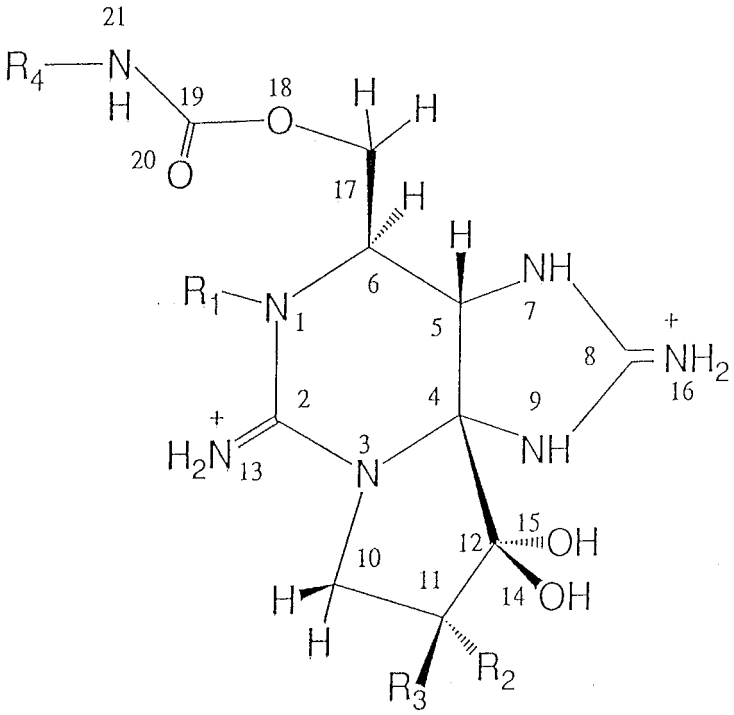

		β	α	
	R1	R3	R2	R4
STX	H	H	H	H
GTX2	H	H	OSO_3^-	H
GTX3	H	OSO_3^-	H	H
B1	H	H	H	SO_3^-
C1	H	H	OSO_3^-	SO_3^-
C2	H	OSO_3^-	H	SO_3^-
NEO	OH	H	H	H
GTX1	OH	H	OSO_3^-	H
GTX4	OH	OSO_3^-	H	H
B2	OH	H	H	SO_3^-
C3	OH	H	OSO_3^-	SO_3^-
C4	OH	OSO_3^-	H	SO_3^-

Fig.1. Structures of the paralytic shellfish poisoning toxins.

to saxitoxin, except for GTX2 which is about 50% that of saxitoxin [Sullivan et al., 1988] indicating similar affinities for nerve cell sodium channel receptors. Although the GTX's are often predominant in toxin profiles of PSP toxin producing algae and contaminated shellfish, they have not been studied as much as saxitoxin which is commercially available. However, pure GTX's are easily isolated in relatively large amounts (1–2 mg/g wet weight of cells) from toxic dinoflagellates by established methods [Laycock et al., 1994]. Selective removal of sulfate from the 21-sulfamates and 11-sulfates of the PSP toxins was described previously [Laycock et al., 1995] and in this report we describe a method which allows selective addition of sulfate to prepare various saxitoxin analogues including radioactive ^{35}S PSP toxins potentially useful in PSP toxin assays.

MATERIALS AND METHODS

Preparation Methods for PSP Toxins From *Alexandrium spp*

Saxitoxin, neosaxitoxin, GTX2, GTX3, C1, and C2 were isolated from *Alexandrium excavatum* Gaspé strain PR 103F as described previously [Laycock et al., 1994]. Neosaxitoxin and C toxins were further purified to remove UV absorbing contaminants on a 1.6 cm (ID) × 32 cm column of Sephadex G-10, equilibrated with water and eluted with a linear gradient of 0–0.05 M acetic acid. GTX1 and GTX4 were isolated as epimeric mixtures from *Alexandrium minutum*, Vigo strain 1. Cells were grown in 30 L carboys or in a 200 L cylinder in f/2 seawater medium [Siegelman and Guillard, 1971]. In order to locate and identify toxins in column fractions, 10 μl samples were spotted across Whatman No. 1 chromatography paper and separated by high voltage paper electrophoresis (HVPE) in 10% acetic acid (3 kV, 30 min) [Laycock et al., 1994]. Thin layer chromatography (TLC) was carried out on silica plates in pyridine, ethyl acetate, acetic acid, water (15:5:3:4) [Buckley et al., 1976]. Toxins were detected by fluorescence under 366 nm UV light after oxidation with 1% H_2O_2 and heating for 5 min. Chromatograms used for autoradiography were exposed to film (Kodak Biomax MR) before spraying.

Radioactive Labelling. Dry methanol (100 μl) was added to 400 μg (1 μmol) lyophylized toxin (GTX's or C toxins) in a 1.7 ml Eppendorf centrifuge tube. The tube was cooled in ice before slowly adding 10 μl acetyl chloride. Analysis by CE/UV showed that sulfate was removed rapidly, but to ensure completion, the reaction mixture was held at 45°C for 30 min. The methanolic solution of the 11-hydroxy derivative was then evaporated to dryness in a vacuum or stream of nitrogen. Carrier free $H_2{}^{35}SO_4$ (Du Pont Canada Inc., Mississauga, ON., Canada) was added to the reaction tube (100 μl containing 1 mCi = 37 MBq) and taken to dryness. Sulfation was carried out at room temperature by adding 10 μl of 0.6 M H_2SO_4 (2 μl 18 M H_2SO_4 in 100 μl dry dimethylformamide) followed by 10 μl 1 M DCC (N,N′-dicyclohexylcarbodiimide, 20 mg in 100 μl dry dimethylformamide) with rapid mixing. If a significant amount of 11-hydroxysaxitoxin was found to remain by CE analysis the reaction mixture was evaporated to dryness in a

vacuum and a further 10 μl of DCC solution was added. The reaction mixture was extracted with water and the solution passed through an anion-exchange cartridge (Waters QMA Sep-Pak) in tandem with a C-18 cartridge (Waters C-18 Sep-Pak).

Analytical Methods

Routine analyses for PSP toxins were performed by capillary electrophoresis using an Applied Biosystems (Santa Clara, CA) model 270A equipped with an uncoated, 70 cm long, 50 μm ID, silica capillary with a window 50 cm from the inlet (anode) end. The electrolyte was 100 mM morpholine formate buffer solution, pH 4.0. A 5 sec injection time was used followed by a separation time of 5 min at 30 kV. The detector was adjusted to 200 nm and Beckman's System Gold (version 3) software was used for data acquisition. The NRC PSP-1 calibration standards (available from the Marine Analytical Chemistry Standards Program, Institute for Marine Biosciences, National Research Council of Canada, Halifax, NS, Canada) were used to convert peak areas to concentrations.

Analyses for C toxins were performed with a Beckman P/ACE System 5510 (Beckman Instruments, Fullerton, CA). Uncoated fused silica capillaries were obtained from Polymicro Technologies (Phoenix, AZ). Dimensions were 50 μm ID, 360 μm OD, and 47 cm long (40 cm to the UV detector). The inner wall of the capillary was conditioned first with 1.0 M NaOH for 20 min, followed by a 5 min rinse with 0.1 M NaOH and a 5 min rinse with water. Under pressure (20 psi), a solution of 5% hexadimethrine bromide in 2% ethylene glycol was rinsed through the capillary for 20 min which was then equilibrated in the running buffer (50 mM sodium phosphate buffer, pH 7.40). The hexadimethrine coating serves to reverse the electroosmotic flow within the capillary [Wiktorowicz and Colburn, 1990] requiring the separations to be done with reverse polarity. A constant voltage of 30 kV was applied across the capillary. Samples were introduced by low (0.5 psi) pressure injection for 10 sec. Separations were monitored using UV detection at 200 nm. To ensure a clean coated surface after each separation as well as reproducible migration times between each separation the capillary was rinsed for 1 min with 0.1 M NaOH followed by 1 min with the hexadimethrine solution and 3 min with the running buffer. Peak identities were confirmed by spiking with standard solutions.

Mass spectra were obtained with a SCIEX (Thornhill, ON., Canada) API III triple quadrupole mass spectrometer. A Bruker AMX-500 NMR spectrometer was used to obtain ^1H NMR spectra at 500 MHz in D_2O. Radioactivity was measured with a Beckman LS 7500 liquid scintillation counter with Ready Safe as the scintillator (Beckman Instruments Inc., Fullerton, CA).

RESULTS

The usual starting material for the sulfation reactions was purified, lyophilized C toxins as these were the most abundant in profiles of our toxic dinoflagellate strains. These doubly sulfated toxins usually accounted for more than 50% of the total PSP toxin content of cold 0.1 M acetic acid extracts of sonicated *Alexandrium excavatum* (Gaspé strain) cells. Mild acid hydrolysis was used to remove sulfate from N-21 as the usual first step. Sulfate was readily removed from the 11-position in methanolic HCl giving mixtures of the two 11-hydroxysaxitoxin epimers with only trace amounts of residual GTX's (Fig. 2A). The sulfation of 11-hydroxysaxitoxin was variable and inefficient until it was found that high concentrations of the reactants were necessary. The best solvent was anhydrous dimethylformamide (Aldrich, Milwaukee, WI) that was sufficiently free of dimethylamine to be undetectable by smell. Optimum reaction conditions were investigated after an initial set of conditions were obtained by trial and error and based on a method for sulfating carbohydrates (Mumma et al., 1970). A solution of 1 μmol of the toxin in 10 μl of DMF was mixed with 10 μl of 0.36 M H_2SO_4 in DMF (3.6 μmol SO_4^{2-}) and concentrated in a vacuum. Sulfation occurred during rapid mixing with 10 μl of 1 M DCC in DMF. The reaction was complete within a few seconds and appeared to solidify with the concomitant production of dicyclohexylurea crystals. Water (80 μl) was then added which precipitated excess DCC as the urea. Yields of the 11-sulfates of saxitoxin were variable until it was discovered that the N-21 position was sulfated during the reaction, especially when a 10 molar excess of sulfate to toxin was adopted. Capillary electrophoresis using hexadimethrine coated capillaries to reverse the electroosmotic flow permitted analysis of the neutral C toxins and revealed the presence of the two epimers, C1 and C2, after sulfation. Furthermore, mild acid hydrolysis of the aqueous reaction mixtures at 100°C for 5 min gave greater and more consistent yields of GTX2 and GTX3.

The optimum molar ratio of sulfate to toxin was investigated in an experiment in which each reaction contained the same amount of 11-hydroxysaxitoxin (0.4 μmol) and different amounts of sulfate (Fig. 3). Endogenous sulfate from the original C toxins provided 0.8 μmol. The graph shows that very little sulfation occurred with no added sulfate. GTX increased in direct proportion to the loss of 11-hydroxysaxitoxin until 0.4 μmol of GTX was formed with 4 μmol of sulfate in the reaction mixture. This tenfold excess of sulfate ensured complete consumption of the 11-hydroxysaxitoxin thus avoiding the necessity to remove the excess by column chromatography. The two major by-products detected by CE at 4.18 min and 4.93 min (Fig. 2B) were produced in control reactions containing no toxin and were probably derived from DCC. These contaminants were removed when the reaction mixture was passed through a C18 cartridge (Fig. 2C).

Labelled GTX's were usually prepared from 1 mg of lyophilized C toxins (2.5 μmol) dissolved in 100 μl of radioactive sulfuric acid solution containing 1 mCi (36 MBq) and 1 nmol $H_2{}^{35}SO_4$. The solution was evaporated to dryness in a vacuum and the residue redissolved in 100 μl of

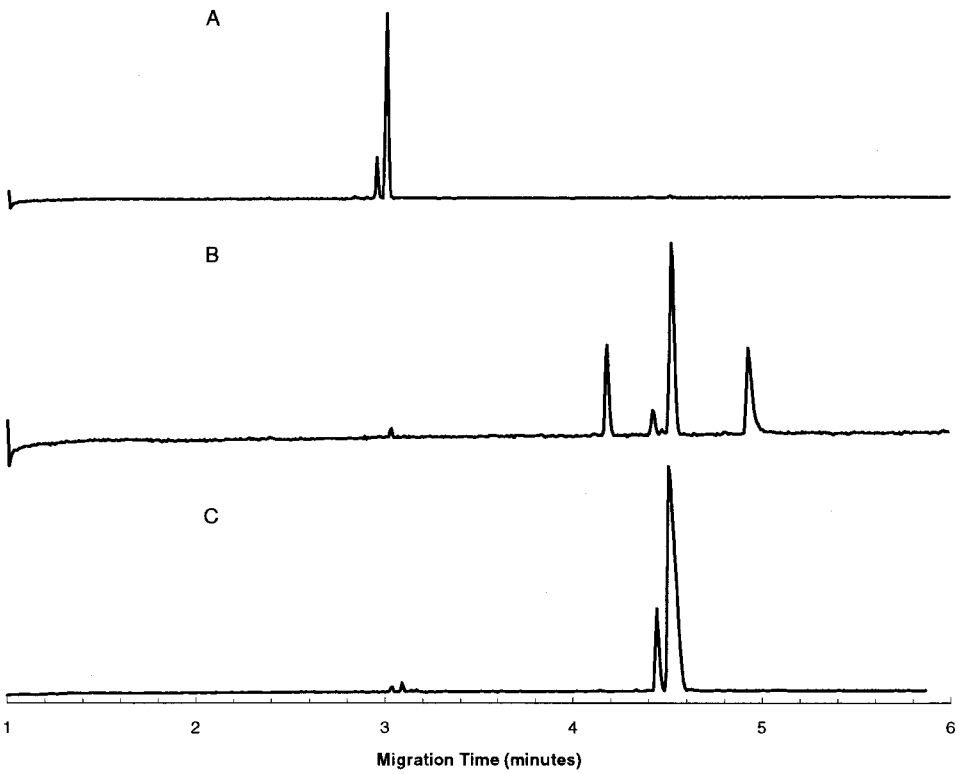

Fig.2. CE/UV analyses of three stages in the preparation of ^{35}S labelled GTX2 and GTX3. **A:** Production of α and β 11-hydroxysaxitoxin epimers from a mixture of GTX2 and GTX3 in methanol containing 10% acetyl chloride. **B:** Sulfation of 11-hydroxysaxitoxins. Migration time for GTX2 was 4.44 min and for GTX3, 4.52 min. Peaks at 4.18 and 4.93 min were reaction products of DCC and SO_4^{2-}. **C:** After purification with C18 and anion-exchange (QMA) SepPaks.

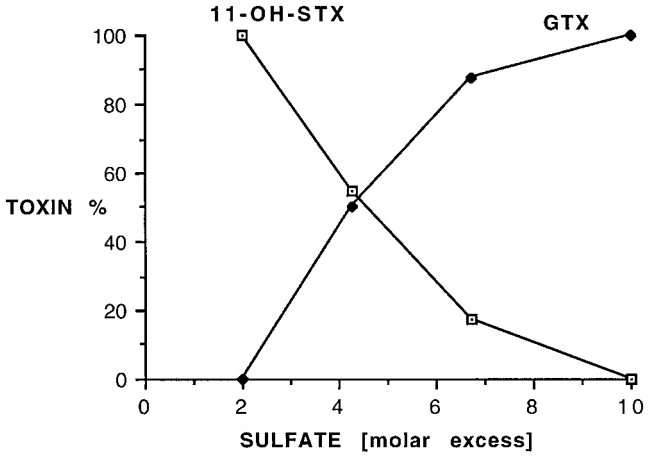

Fig.3. Effect of sulfate concentration on the yield of GTX. Each reaction contained 0.4 μmol of 11-hydroxysaxitoxin to which was added 0, 2, 5, 10, and 20 μl aliquots of a 0.18 M solution of H_2SO_4 in DMF followed by 10 μl of a 1 M solution of DCC in DMF. Each reaction mixture was diluted to 100 μl with water and kept at 100°C for 5 min. Samples were analysed for GTX by CE.

anhydrous methanol. The solution was cooled in ice before adding 10 μl of acetyl chloride. After 15 min at 50°C CE analysis showed complete conversion to the two 11-hydroxysaxitoxin epimers (Fig. 2A). To obtain labelled toxins with relatively high specific activities the amount of unlabelled sulfate was reduced but at the expense of a low yield as the molar ratio of sulfate to toxin was below the optimum ratio of 10:1 indicated in Figure 3. After addition of 10 μl of 1 M DCC in DMF the reaction mixture was dissolved in water and kept at 100°C for 5 min to hydrolyze sulfate from the 21-position. Autoradiograms of the reaction mixture without additional sulfate showed numerous by-products that were not seen when a greater excess of sulfate was present. These included a doubly sulfated by-product which was not altered by mild acid hydrolysis. On TLC autoradiograms this acidic by-product moved ahead of GTX2 and GTX3. Autoradiography after paper electrophoresis showed that excess sulfate and the acidic by-product were completely removed with an anion exchange cartridge. The labelled toxin was purified by column chromatography on BioRex-70 (H^+ form) with isocratic elution in 0.05 M acetic acid. With molar ratios of around 3:1 (sulfate:toxin) the recovery of labelled GTX 2/3 was about 5% of the starting toxin with specific activities of 50–55 μCi/mmol (1.8–2.0 MBq/μmol). With 10:1 molar ratios yields were about 50% but specific activity fell in proportion to the dilution of the radioactive sulfate so that GTX specific activities were 15–20 μCi/μmol (0.5–0.7 MBq/μmol). The highest specific activity was obtained with 1 nmol of toxin, 1

mCi $H_2{}^{35}SO_4$ and 7 nmol H_2SO_4. The specific activity of added sulfate was 10^5 mCi/μmol (3.6 GBq/μmol) but that of the GTX after the sulfation reaction was only 130 μCi/μmol (4.7 MBq/μmol).

The identity of radioactive products was confirmed by co-chromatography with GTX2/3 standards. Paper electrophoresis showed that the radioactive compounds were positively charged and electrophoretic mobilities were identical to those of GTX2 and GTX3. These two epimers did not separate from each other by paper electrophoresis but were well separated on thin layer plates. GTX2 and GTX3 standards were detected on TLC plates by fluorescence of their oxidation products and these spots were superimposable on the radioactive compounds detected by autoradiography (Fig. 4). Both epimers were produced by the sulfation reaction but the relative intensities of labelling depended on the starting material. Only two peaks were detected by CE/UV (Fig. 2C) in the final preparation and these were indistinguishable from GTX2 and GTX3 standards. The proton NMR spectrum of sulfated 11-hydroxysaxitoxin was identical to that of GTX2/3 isolated from dinoflagellates. The ionspray mass spectrum (Fig. 5) is typical of GTX2/3 with a protonated molecule at m/z 396 and fragment ions at 378 (loss of H_2O), 316 (loss of SO_3), and 298 (loss of SO_3 and H_2O).

Sulfation of the N-21 position was investigated further using the optimum reaction conditions for further sulfation of 11-sulfate esters. With GTX1/4 more than half of the toxin was first converted to 11-hydroxyneosaxitoxin and the radiolabel was incorporated into both GTX1/4 and C3/4 toxins. Six derivatives were detected on TLC plates indicating that the N-1 hydroxyl group was also partially sulfated. Sulfation of GTX2/3 produced some 11-hydroxysaxitoxin in addition to radiolabelled GTX2/3 and C1/2. With saxitoxin there was only one radiolabelled product and this was identical to B1 toxin by CE, TLC, and HVPE.

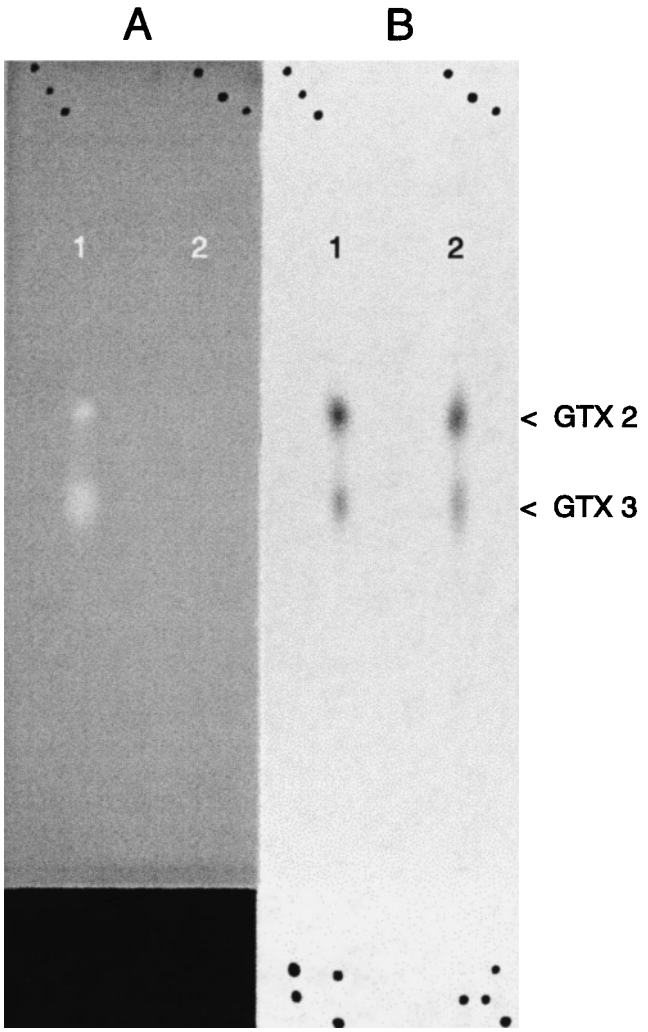

Fig.4. Identification of ^{35}S labelled gonyautoxins by autoradiography on a silica TLC plate. Two spots (1 and 2) of purified labelled GTX2 and GTX3 prepared from C1 and C2 toxins were applied to the plate which was then developed in the mobile phase; pyridine, ethyl acetate, water, acetic acid, (15:5:4:3). In the photograph of the TLC plate on the left (**A**) GTX2 and GTX3 appear as fluorescent spots in **lane 1** to which a mixture of purified GTX2 and GTX3 was added as markers The TLC plate was photographed under long wavelength UV light after spraying with 1% H_2O_2 solution then heated at 100°C for 5 min. All of the radioactivity detected on the autoradiogram (**B**) of the TLC plate was associated with the GTX spots. Radioactive ink spots, seen at the top and bottom were used to align the plate and autoradiogram. Note: The marker solution contained a higher proportion of GTX3 than GTX2, which is not related to ratio of the radiolabelled epimers seen in the autoradiogram (B) where GTX2 is predominant.

DISCUSSION

It was shown earlier [Laycock et al., 1995] that sulfate can be completely removed from GTX's in methanolic HCl leaving -OH at the 11-position. This simple and efficient reaction for the complete removal of sulfate from GTX2/3 to prepare 11-hydroxysaxitoxin greatly facilitated the investigation of a method for sulfation and incorporation of radiolabel into PSP toxins. It was found later that it is not essential to remove sulfate and substantial incorporation of radiolabel occurred without this first step. Natural PSP toxins can be sulfated at two sites in the molecule: a) in the side chain on N-21, giving rise to the sulfamate toxins, and b) in the gonyautoxins as sulfates at C-11 as **α** or **β** epimers (Fig. 1). Biosynthetic sulfation of -OH and $-NH_2$ groups is mediated by sulfotransferases with an activated carrier, usually 3′-phosphoadenosine 5′-phosphosulfate (PAPS). For the biosynthesis of PSP toxin sulfate esters the substrate is most probably 11-hydroxysaxitoxin. Sulfotransferase activity was not detected in homogenates of dinoflagellate cells with added ^{35}S labelled PAPS and 11-hydroxysaxitoxin, due largely to the rapid enzymatic hydrolysis of sulfate and phosphate from PAPS. Also, attempts to incorporate radioactivity into PSP toxins by growing cells in media containing $^{35}SO_4{}^{2-}$ were unsuccessful.

Sulfation by synthetic methods is achieved by shifting the equilibrium of the reaction equation to the right with an excess of sulfate and loss of water:

$$R—OH + H—O—SO_3H \rightleftharpoons R—O—SO_3H + H_2O$$

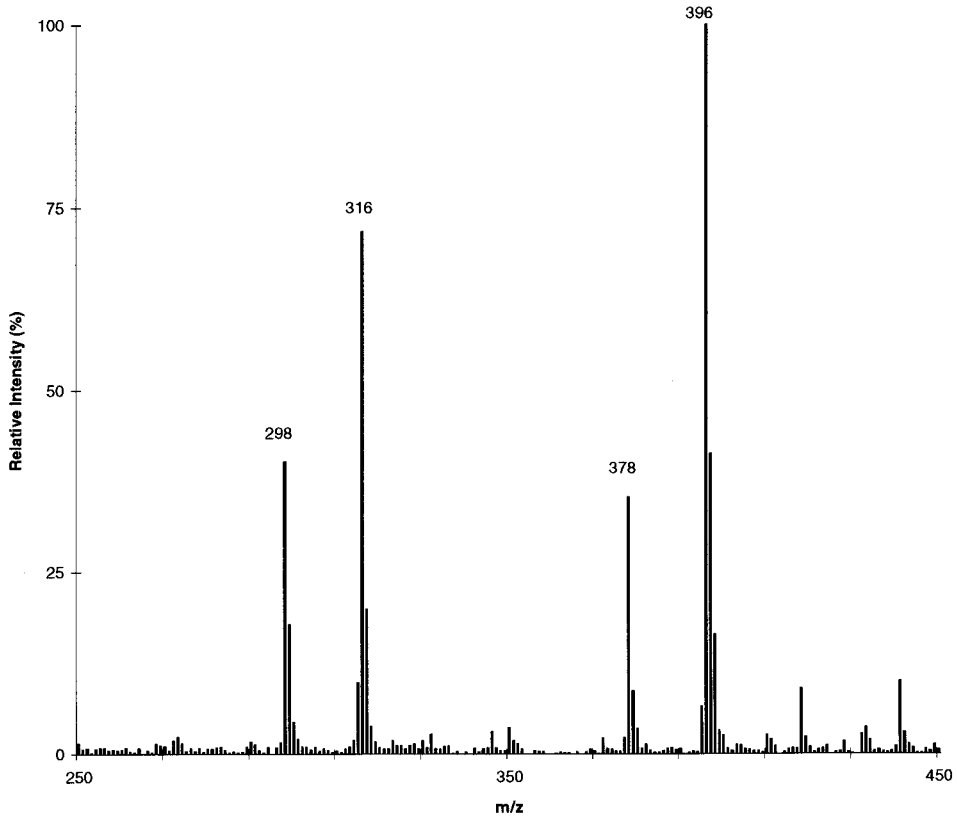

Fig.5. Ionspray mass spectrum of a purified partially ^{35}S labelled toxin preparation showing ions of GTX2 and GTX3 at m/z 396 corresponding to $(M+H)^+$ 378 $(M+H-H_2O)^+$, 316 $(M+H-SO_3)^+$ and 298 $(M+H-SO_3-H_2O)^+$. Fragment ions at 316 and 298 are also partly due to ionization of a small amount of residual 11-OH-STX which gives the same ions (see Fig. 2C).

This reaction is commonly carried out with sulfur trioxide-pyridine complex [Lloyd, 1960; Peat et al., 1960] or H_2SO_4 and N,N′-dicyclohexycarbodiimide (DCC) in suitable anhydrous solvents [Mumma et al., 1970]. Because $H_2{}^{35}SO_4$ is relatively inexpensive and of high specific activity the DCC method was investigated for the preparation of radioactive GTX's. The large quantities of reactants used in earlier work on sulfation of carbohydrates [Mumma et al., 1970] could not be used but high toxin concentrations were found to be essential for good recoveries. Reaction volumes of 10 to 20 μl were optimal with 1 μmol of toxin. Pure anhydrous dimethylformamide dissolved 11-hydroxysaxitoxin, H_2SO_4, and DCC to the required concentrations but a high degree of purity of the solvent is essential. Purification of DMF is not simple [Perrin et al., 1980], however, anhydrous DMF of adequate purity is available from Aldrich. Mumma et al. [1970] emphasized the importance of the order in which the reactants are mixed (DCC + hydroxy derivative + H_2SO_4) and that molar ratios of at least 5:1 DCC to hydroxy derivative and not more than 1.5:1 excess sulfate to alcohol with all reactants at O°C, gave the best yields for gram quantities of carbohydrates. Possibly due to physical differences in mixing very small volumes our best yields were obtained when 11-hydroxysaxitoxin and H_2SO_4 were mixed and dried to a film before adding the DCC solution. The reaction was essentially instantaneous whether it was at room temperature or in ice. Molar ratios of less than 1.5 sulfate to toxin gave poor yields and a tenfold excess was necessary for complete sulfation of 11-hydroxysaxitoxin to GTX (Fig. 3).

For applications such as radioimmunoassays and receptor site binding assays sensitivity depends on specific activity. Tritiated saxitoxin prepared by exchange with carrier free tritiated water can have a specific activity of 40 μCi/μmol (1.48 GBq/μmol) [Ritchie et al., 1976]. The specific activity of carrier free $H_2{}^{35}SO_4$ is 1.5 Ci/μmol (54 GBq/μmol). It should be possible, therefore, to prepare labelled GTX's with much greater specific activities than the 50 μCi/μmol often obtained in our experiments. An attempt to prepare high specific activity GTX2/3 with a moderate amount of radioactivity (1 mCi) and a 10:1 ratio of sulfate to toxin and only 1 nmol of toxin resulted in 130 μCi/μmol GTX, well below the calculated specific activity of sulfate (10^5 μCi/μmol) added to the reaction. Larger total amounts of ^{35}S may be necessary to achieve a product of significantly higher specific activity. Radioisotope licenses rarely allow the large amounts of radioactivity necessary to achieve labelled GTX with specific activities of greater than 10^3 μCi/μmol, but this could be done by one of the manufacturers of isotopically labelled compounds.

The sulfation reaction described here can also be used to prepare PSP toxin analogues that are not commonly avail-

able from natural sources. Sulfation of STX, for example gave the sulfamate B1 (GTX5) in good yield with no by-products. Similarly, a single labelled product was obtained from decarbamoylsaxitoxin by sulfation of the hydroxyl group on C-17. B2 (GTX6) was also prepared by this method but the N-1 hydroxyl group was also sulfated resulting in a lower yield and a column purification step to separate the three products. Sulfation of GTX1/4 released a surprising amount of 11-hydroxyneosaxitoxin and six labelled products with sulfate at 1, 11, and 21 positions (GTX1, GTX4, GTX1+N-21, GTX1+N1, GTX4+N-21, GTX4+N-1). With three sulfate groups the overall charge would have been negative during paper electrophoresis in 10% acetic acid, but no negatively charged toxins were detected on electrophoretograms by either radioactivity on autoradiograms or by fluorescence after oxidation. The absence of triple sulfates is probably due to steric hindrance of sulfate groups which occupy a large volume with their counterions and water of hydration. The release of 11-hydroxyneosaxitoxin is a hydrolytic reaction and indicates that the reaction mixture was not rigorously anhydrous. Four labelled products of sulfation of GTX2/3 (GTX2, GTX3, C1, and C2) were identified together with a small amount of 11-hydroxysaxitoxin. Sulfamates of the 11-hydroxy toxins were not detected from either GTX1/4 or GTX2/3.

The ability to interconvert the various PSP toxins by selective removal and addition of sulfate makes a wider range of the group more readily available as standards for the development of better analytical methods. The double sulfates of neosaxitoxin (C3 and C4) are rarely found in shellfish or algal sources but they can now be prepared from GTX1 and GTX4. Sulfation can be used to prepare B1 (GTX5) if saxitoxin is more readily available than C1 and C2 toxins from which B1 can be made by thiol reduction [Laycock et al., 1995]. Perhaps the most valuable use of the sulfation reaction is for the preparation of radiolabelled toxins which can be used in, or in the development of, assay methods. Until recently many of the PSP toxins were not available and assay methods were strongly biased towards saxitoxin. It has become increasingly apparent that such methods can be misleading because saxitoxin is not as common as was once thought. The gonyautoxins are by far the most prevalent of the PSP toxins and a greater emphasis on these sulfate derivatives would lead to more reliable methods of detection. The production of antibodies to saxitoxin and neosaxitoxin has been reported but none, so far, to the gonyautoxins. Radiolabelled ^{35}S toxins have been especially useful in the preparation of GTX conjugates. For the first time it is possible to directly measure the amount of toxin bound to each protein molecule of a conjugate. Rapid exchange of the label with water makes this impossible with tritiated saxitoxin.

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